

Ultrastrukturelle Veränderungen hervorgerufen durch das Cancerogen Aflatoxin B₁ in verschiedenartigen Geweben

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Ultrastructural Changes Produced by the Carcinogen, Aflatoxin B₁, in Different Tissues

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Summary. In the present comparative study, tissues differing widely in structure and function were exposed to acute concentrations of the carcinogen, aflatoxin B₁, and examined by electron microscopy. It was found that the most prominent ultrastructural change—the segregation and zoning of nucleolar components—followed an essentially identical pattern in both fish hepatic cells and ciliated molluscan epithelial cells and closely resembled the corresponding nucleolar reorganization in rat hepatic parenchymal cells. The biochemical lesions reflected by these nucleolar changes may apparently be induced in eukaryotic cells in general and are not tissue-specific. No neoplasms were observed in either fish or mollusks surviving an LD₅₀ of aflatoxin B₁ which has been demonstrated to produce a high incidence of hepatic carcinomas in rats.

Introduction

The aflatoxins are a group of closely related metabolites produced by the fungus *Aspergillus flavus* and certain other mold species (Diener and Davis, 1969). In addition to a high level of toxicity for both animals and plants (Wogan, 1966), the compounds have been shown to be potent hepatocarcinogens in experimental animals (Lancaster *et al.*, 1961; Wogan and Newberne, 1967; Carnaghan, 1967).

In rat hepatic cells, alterations of fine structure produced by acute administration of aflatoxin B₁ are characterized by the segregation and zoning of nucleolar components closely resembling the nucleolar changes produced by other carcinogens such as 4-nitroquinoline-N-oxide, lasiocarpine, and 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB). There is strong evidence to indicate that this type of nucleolar reorganization is the specific effect of substances binding to DNA and interfering with RNA synthesis (Simard and Bernhard, 1966) while a relationship with subsequent tumor development has yet to be established (Reddy and Svoboda, 1968).

Although liver injury is the principal effect of aflatoxin B₁ intoxication, and the results of autoradiographic studies seem to suggest a specificity of its inhibitory action on RNA and protein synthesis in hepatic cells, leaving fibroblasts unaffected (Zuckerman *et al.*, 1968), it was felt that if inhibition of basic nucleic acid metabolism is indeed the primary biochemical lesion produced by aflatoxin B₁ (Clifford and Rees, 1966; Sporn *et al.*, 1966) eukaryotic cells in general should be susceptible.

In the present comparative study, two structurally and functionally dissimilar tissues—hepatic parenchyma of the small aquarium fish *Brachydanio rerio* and the ciliated epithelium covering tentacles of the fresh water snail *Planorbarius corneus*—were examined by electron microscopy following acute exposure of the animals to aflatoxin B₁. Both species were found to be unsusceptible to the carcinogenic action of the agent under conditions of acute administration comparable to those employed by Carnaghan (l.c.) to produce hepatic carcinoma in rats.

Material and Methods

The Zebra danio, *Brachydanio rerio*, is a hardy fresh water species which is readily maintained in small aquarium tanks on a diet of commercial dry food. Grown specimens were obtained from a local breeder and kept in aerated aquariums for three weeks prior to the experiments. Every other day, the fish were fed with TetraMin consisting mainly of fish meal.

Aflatoxin B₁¹ was added to the water of experimental tanks dissolved in propylene glycol, the solvent in the aqueous solution not exceeding 0.1%; alternatively, the agent was dissolved directly in water by heating an aqueous suspension of the dry substance near the boiling point for 30 minutes. Equivalent amounts of the solvent were added to some of the control tanks. No measurements were made of the disappearance of aflatoxin from the water. However, aqueous solutions of aflatoxin have been found to be relatively stable (Tauchmann and Leistner, 1969).

The fish were exposed in three groups of 30 specimens each to a 2 ppm solution of aflatoxin B₁ (= 2 µg/ml) which was lethal for approximately 50% of the animals following 3 days of treatment. Ten specimens each were sacrificed by decapitation after 24, 48, and 72 hours of exposure, resp., and their livers removed for electron microscopy. Ten surviving fish of each exposure group were returned to control tanks without aflatoxin and examined 9 months later for neoplastic growths.

Planorbarius corneus is a small pulmonate fresh water snail commonly found in ponds. Mature specimens were obtained from a zoological supply store and held in aerated tanks for several weeks prior to the experiments. The snails thrived on a diet of TetraMin. Addition of aflatoxin B₁ to the experimental tanks followed the methods described above. The snails were kept in a 2 ppm aqueous solution of aflatoxin B₁ for 24 hours or in an 0.3 ppm solution for 48 hours, resp. At the higher concentration, all animals not scarified died within 3 days from the beginning of treatment, while at the lower concentration 50% of the specimens succumbed to the toxin within 8 days following termination of the exposure. After 9 months, 50 surviving snails of the 0.3 ppm exposure group were dissected and examined for neoplastic growths.

Electron Microscopy. Livers of *B. rerio* were minced into small cubes and fixed for 20 minutes in glutaraldehyde (3%, in 0.135 M phosphate buffer, pH 7.2). They were postfixed for one hour in osmic acid (1%, in 0.135 M phosphate buffer, pH 7.2), dehydrated in a graded series of alcohols, and embedded in Durecupan ACM.

Tentacles of *P. corneus* were removed with fine scissors and divided into segments 1 mm in width. Fixation and embedding followed the procedure described above.

¹ Makor Chemicals, Jerusalem, Israel.



Thin sections were cut with a Reichert ultramicrotome, mounted on copper grids, and stained with uranyl acetate and lead citrate (Reynolds, 1963). They were examined with a Siemens Elmiskop 101 electron microscope at an accelerating voltage of 80 kV.

Results

I. Hepatic Cells of B. rerio

Controls. The fine structure of these cells was closely similar to that of rat parenchymal liver cells. Mitochondria were oval or elongate, with a homogeneous matrix and some electron-dense granules. The rough endoplasmic reticulum was well developed and occurred as stacks of double membranes close to the nucleus. Aggregates of glycogen granules were regularly present.

The nucleus possessed one or two nucleoli composed of granules measuring 150–200 Å in diameter as well as fibrillar structures embedded in an homogeneous matrix. Islands of condensed chromatin were found along the inner surface of the nuclear membrane and scattered throughout the nucleoplasm.

Aflatoxin B₁, 24 Hours Exposure. The most prominent and consistent changes were observed in the nuclei of hepatic cells. Under the action of aflatoxin B₁, the large and loosely organized nucleoli with irregular outlines characterizing normal hepatocyte nuclei showed a marked decrease in size and had assumed a compact, spheroidal shape (Fig. 1). Two zones of different densities were usually apparent in these modified nucleoli, with a zone of moderate density being composed largely of fibrils approximately 50 Å in width and granular elements predominating in the zone of higher electron-density (Figs. 2, 3). The appearance of these nucleoli was thus highly suggestive of the segregation and zoning of the components of normal nucleoli.

A characteristic of the fibrillar zone were vacuoles containing clusters of electron-opaque granules measuring 360–400 Å in diameter and exhibiting marked similarity in size and shape (Fig. 2). Aggregates of similar granules were frequently found close to nucleoli and appeared to be surrounded by low-density material.

Cytoplasmic structures were little changed. The rough endoplasmic reticulum was less regularly arranged, but there was no loss of ribosomes from its membranes. Occasionally, hypertrophied mitochondria were observed, and there was an obvious decrease in the density of glycogen aggregates.

Aflatoxin B₁, 48–72 Hours Exposure. By the rounding off of the granular and fibrillar zones, the separation of nucleolar components had become more pronounced in many hepatic cells following extended exposure (Fig. 3). Frequently, the zones were found to be completely detached, lying in the nucleoplasm at some distance from each other (Fig. 4). With the progressive separation of nucleolar components, the granular elements had become markedly coarser, while the nucleoplasm showed a loss of density.

In the cytoplasm, there was now a proliferation and rearrangement of the rough ER in the form of concentric whorls of lamellae (Fig. 5). Some mitochondria appeared slightly swollen with flocculant matrix material, while others contained electron-transparent membrane-bound vacuoles or exhibited various degrees

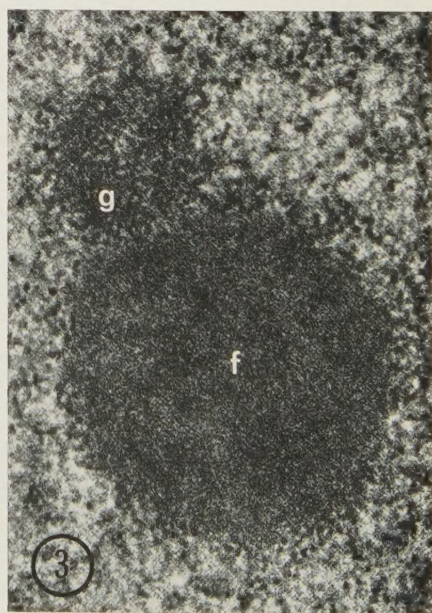
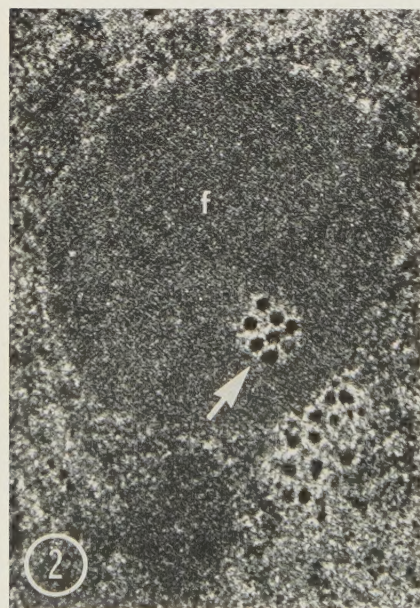
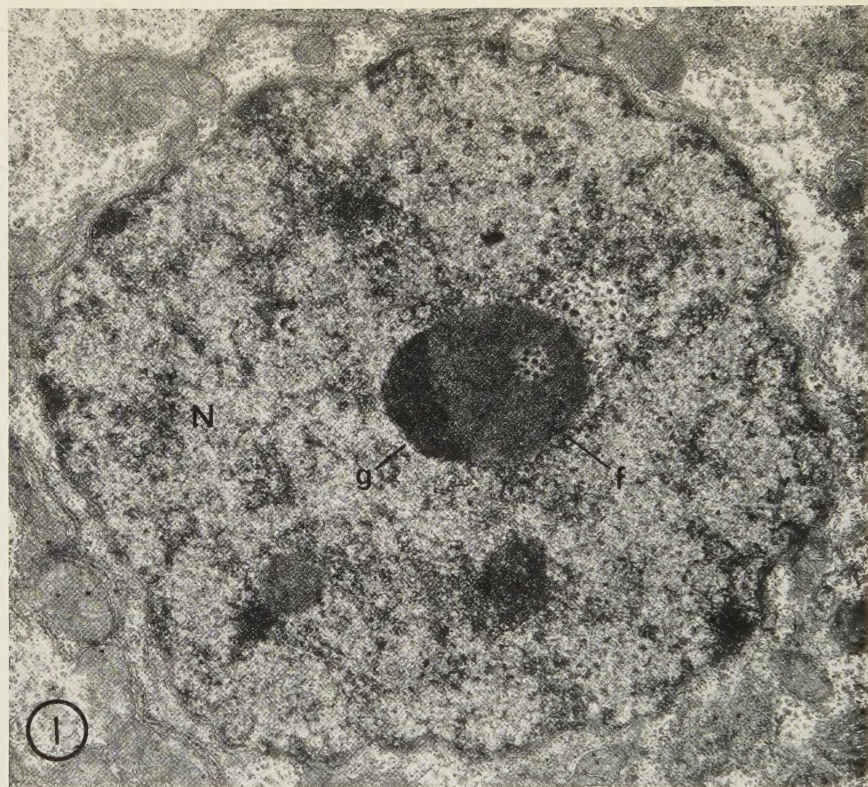


Fig. 1—3

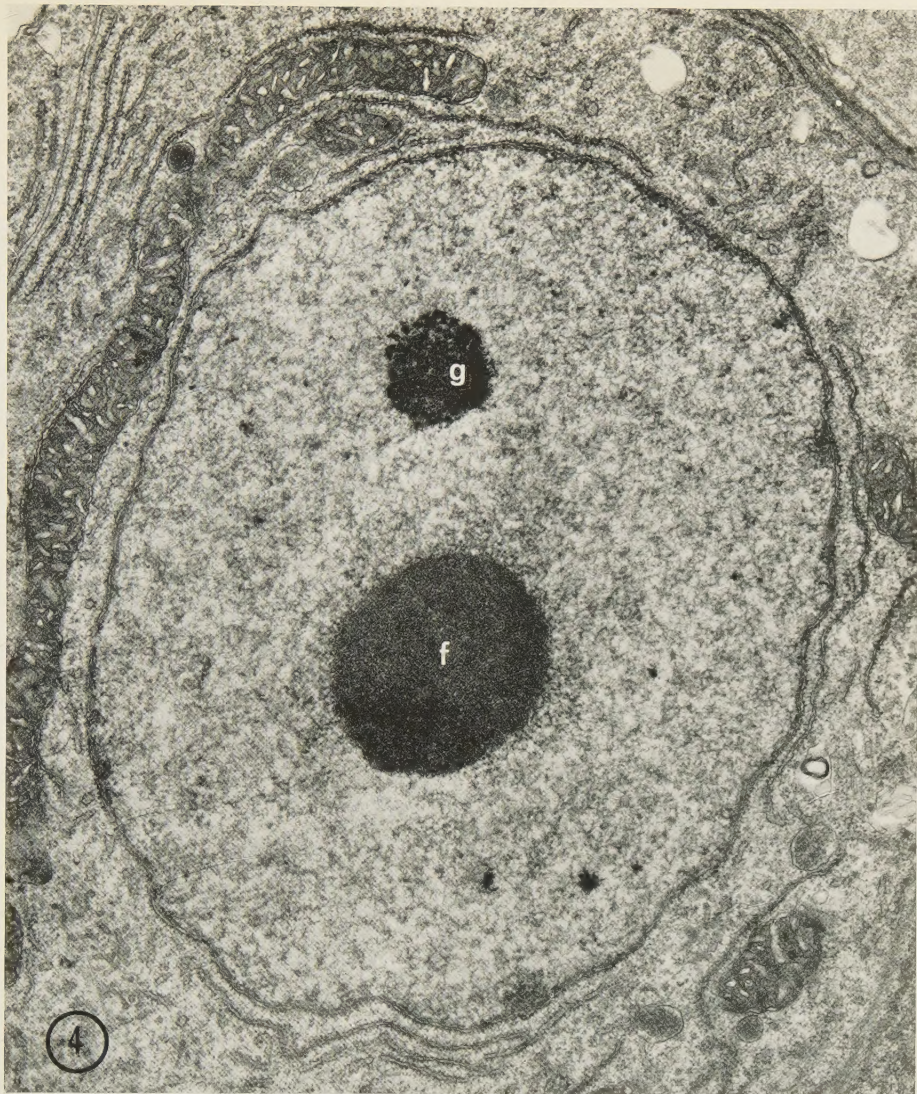


Fig. 4. Complete spatial separation of the granular (*g*) and fibrillar (*f*) zones of hepatocyte nucleoli was frequently seen at 72 hours exposure to aflatoxin B₁. $\times 22000$

Fig. 1. Hepatocyte nucleus (*N*) of *Brachydanio rerio* following 48 hours of acute exposure to aflatoxin B₁. The nucleolus is smaller and more compact than normal and exhibits a dense zone (*g*) and a zone of moderate density (*f*) containing a vacuole with opaque granules. $\times 18000$

Fig. 2. The moderately dense nucleolar zone (*f*) is seen to be composed of fibrillar material in an homogeneous matrix. Large spherical granules contained in a vacuolar space ($\rightarrow$) are remarkably uniform in size and shape. Similar granules are seen to lie adjacent to the nucleolus. 48 hours exposure to aflatoxin B₁. $\times 42000$

Fig. 3. By rounding off of the nucleolar zones, more advanced separation of granular (*g*) and fibrillar (*f*) components was apparent following 72 hours of exposure to aflatoxin B₁. $\times 50000$



Fig. 5. Whorls of concentric membranes formed by proliferation of the rough endoplasmic reticulum (*ER*) were the most prominent cytoplasmic change in fish hepatocytes following 48–72 hours exposure to aflatoxin B₁. Some mitochondria (*M*) exhibit moderate swelling and loss of matrix material. *S* sinusoid; *N* nucleus. $\times 14000$

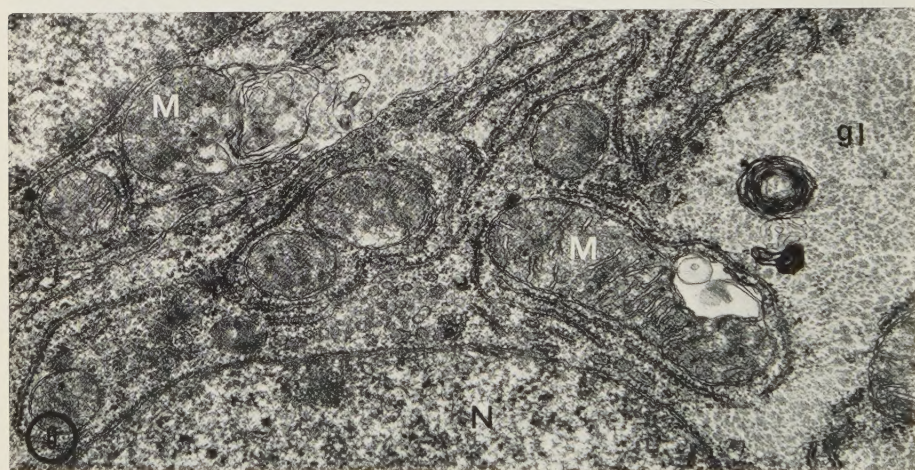


Fig. 6. Mitochondrial changes in hepatocytes were frequently marked by vacuolar degeneration. *gl* glycogen. $\times 28000$

Fig. 7. Adjacent tentacle epithelial cells of *Planorbarius corneus*. (cell borders $\rightarrow$). Following exposure to aflatoxin B₁ (24 hours, 2 ppm) the nucleoli of many cells showed a striking separation into a dense granular (*g*) and a fibrillar (*f*) zone. *N* nucleus; *Go* Golgi apparatus; *Cy* cytolysosomes; *My* myelin figure in cytolysosome. $\times 25000$

Fig. 8. Early phase of detachment of granular (*g*) and fibrillar (*f*) zones. Tentacle epithelial cell, 24 hours exposure to 2 ppm aflatoxin B₁. $\times 35000$

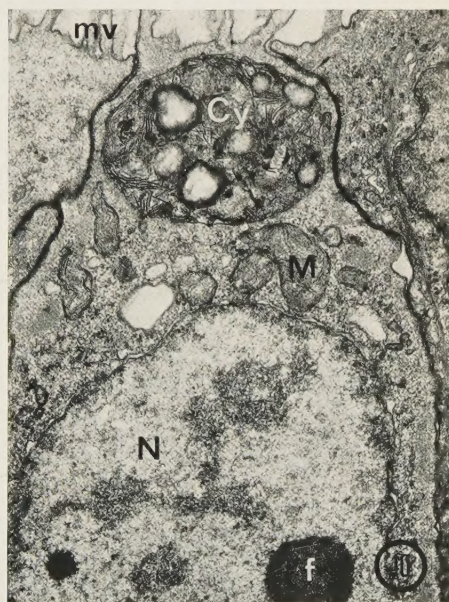
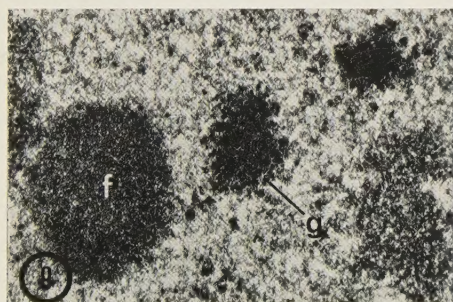
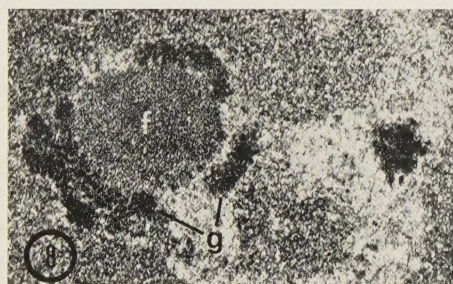
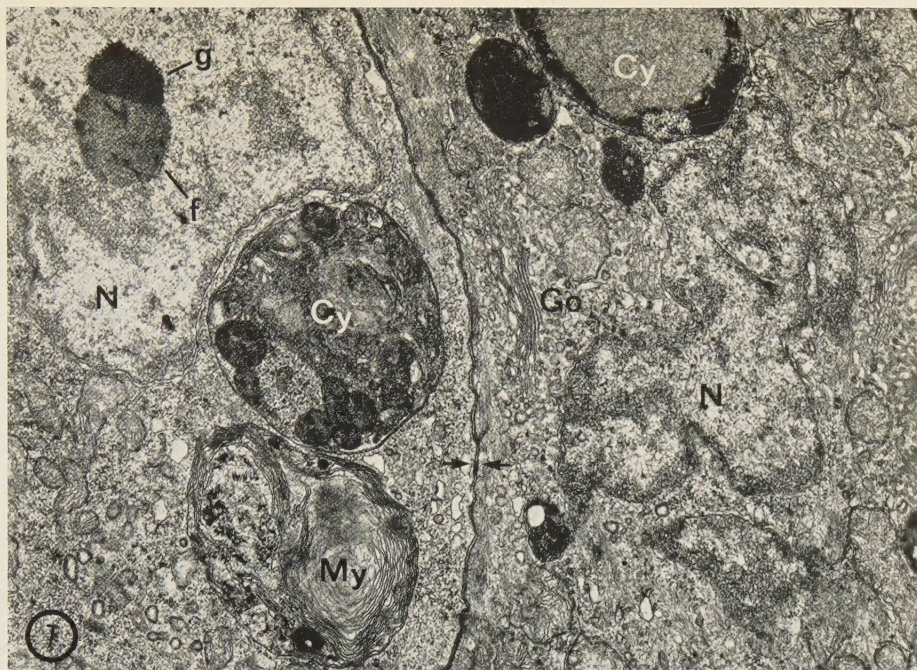


Fig. 9. Complete spatial separation of fibrillar (*f*) and granular (*g*) nuclear material. Tentacle epithelial cell, $\times 35000$

Fig. 10. Loss of nuclear density is obvious following 24 hours exposure to aflatoxin B_1 . Tentacle epithelial cell. *f* fibrillar nucleolar material; *M* mitochondrion; *mv* microvilli; *Cy* cytolysosome. $\times 25000$

of cristolysis (Fig. 6). Occasionally, areas of focal cytoplasmic necrosis containing partially sequestered mitochondria and rough ER were observed.

II. Tentacle Epithelial Cells of *P. corneus*

Controls. The simple epithelium covering the tentacles of this molluscan species was composed of columnar ciliated cells with microvilli at their free cell border. Endoplasmic reticulum was sparsely developed, but there were many free ribosomes and prominent Golgi areas. The nuclei of these cells contained one large nucleolus composed of peripheral granules and a central portion of fibrillar elements.

Aflatoxin B₁. In either treatment group, there was a considerable range within the same tentacle from epithelial cells with a high degree of injury to cells with only moderate alterations. In cells with lesser damage, as evidenced by their over-all density, the nucleoli were usually composed of two hemispheres of unequal size attached at their broad bases (Fig. 7). As in the hepatocyte nucleoli, these hemispherical zones were composed of granular and fibrillar structures, respectively, with the granules characterized by high electron-density (Fig. 7). Frequently, the material of the granular zone was fragmented and partly or completely detached from the fibrillar component (Figs. 8, 9). The pattern of nucleolar changes thus bore a striking resemblance to that observed in hepatic cells of *B. rerio*.

Cytoplasmic changes were characterized by the abundant occurrence of membrane-bound inclusions containing cytoplasmic organelles in various stages of degradation (Figs. 7, 10). Microvilli showed only occasional blebbing but more often appeared unaltered, although they were directly exposed to the aflatoxin solution.

No neoplastic growths were observed in either *B. rerio* or *P. corneus* examined 9 months after acute exposure to aflatoxin B₁.

Discussion

The present study demonstrates that segregation and zoning of nucleolar components produced by acute exposure to the carcinogen, aflatoxin B₁, in hepatic cells of a fish (*Brachydanio rerio*) and tentacle epithelial cells of a mollusk (*Planorbarius corneus*) are essentially identical to the nucleolar reorganization seen in rat hepatic cells following acute administration of the compound (Bernhard *et al.*, 1965; Svoboda *et al.*, 1966; Butler, 1966). Obviously, nucleolar zoning is a phenomenon which may be evoked by aflatoxin B₁—intoxication in functionally and structurally markedly diverse cell types.

Simard and Bernhard (1966), studying the effects of various antimetabolites, have suggested that segregation and zoning of granular and fibrillar nucleolar structures is a characteristic alteration produced by substances that bind with DNA and interfere with its template activity in DNA-directed RNA synthesis. Binding of aflatoxin B₁ to DNA has been demonstrated *in vitro* (Clifford and Rees, 1966), and since interference with the DNA-RNA axis, as reflected by inhibition of RNA- and protein synthesis, has been thought to be responsible

for aflatoxin B₁-induced nuclear zoning in rat hepatic cells (Svoboda *et al.*, l.c.) it is reasonable to suppose that the identical nucleolar changes observed in the present investigation are due to a similar biochemical lesion.

Our findings would thus disagree with the conclusion reached by Zuckerman *et al.* (1967) from their autoradiographic studies with mixed cell cultures that the cytotoxic action of aflatoxin B₁ is specific for hepatocytes. It may be that the different metabolic rates of hepatocytes and fibroblasts, resp., account for the failure of aflatoxin B₁ to inhibit RNA- and protein synthesis in the latter cell type under the experimental conditions used.

Besides aflatoxin B₁, the carcinogens 4-nitroquinoline-N-oxide (Reynolds *et al.*, 1963), 3'-methyl-4-dimethylaminoazobenzene (Floyd *et al.*, 1968), tannic acid (Racela *et al.*, 1967), dimethylnitrosamine and lasiocarpine (Reddy and Svoboda, 1968), ultraviolet light (Montgomery *et al.*, 1966) and the mutagens acridine organe and proflavine (Reynolds and Montgomery, 1967) are among the agents producing nucleolar segregation. The results of the present study indicate, however, that tumor induction is no necessary consequence of this type of nucleolar rearrangement, since no neoplasms were observed in either *B. rerio* or *P. corneus* following exposure to aflatoxin B₁ under acute conditions comparable to those employed by Carnaghan (l.c.) to produce hepatic tumors in rats. Also, antimetabolites not known to be carcinogenic such as actinomycin D (Reynolds *et al.*, 1964) and mithramycin (Kume *et al.*, 1967) have been shown to produce similar nucleolar changes in hepatic cells.

The formation of dense 'macrogranules' in the fibrillar zone of reorganized nucleoli as observed in this study in hepatocytes of *B. rerio* is of special interest. These granules differ from the densities induced by aflatoxin B₁ in the nucleoli of rat and monkey liver cells (Bernhard *et al.*, l.c.; Svoboda *et al.*, l.c.) and from the dense nucleolar plaques seen in Chang liver cells after treatment with actinomycin D or ultraviolet nuclear irradiation (Reynolds *et al.*, l.c.; Montgomery *et al.*, l.c.). By their remarkably regular shape and their characteristic aggregation in small clusters contained in vacuolar spaces, these granules are also unlike the 'microspherules' found in nucleoli of tumor cells following administration of actinomycin D or 4-nitroquinoline-N-oxide, resp. (Unuma and Busch, 1967; Floyd *et al.*, l.c.) and the dense fibrillar nodules described by Recher *et al.* (1971) in nucleoli of ME-180 tissue culture cells after treatment with proflavine. It is anticipated that future elucidation of the chemical nature of the present 'macrogranules' may aid in the further clarification of chemical events underlying the zoning of nucleolar components.

The most prominent cytoplasmic change produced by aflatoxin B₁ in hepatic cells of *B. rerio* was the proliferation of the endoplasmic reticulum with the formation of concentric whorls of ribosome-studded membranes, which may be considered to be an adaptive reaction of the cell to compensate for inhibition of protein synthesis. However, similar whorls observed by Svoboda *et al.* (l.c.) in aflatoxin-injured rat liver cells were of the smooth type, lacking the ribosomes functioning in protein synthesis. It appears that the proper interpretation of the significance of cytoplasmic alterations following exposure to aflatoxin B₁ must await further study of the question whether there is some direct effect on cyto-

plasmic organelles, as proposed by Theron (1965), or whether disturbance of nuclear metabolism is the principal biochemical lesion producing both nuclear and cytoplasmic fine-structural changes.

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Lebenslauf

Am 21. Juli 1945 wurde ich als erstes Kind des Finanzbeamten Tjio Kong Hoey und seiner Ehefrau Betsy Thio in Djakarta (Indonesien) geboren.

1951 wurde ich in Djakarta in der Volksschule eingeschult.

Ab 1956 besuchte ich die mittlere Oberschule und ab 1959 die Kanisius-Oberschule. 1963 legte ich dort das Abitur ab.

1963 bis 1966 studierte ich Medizin an der Universität Trisakti in Djakarta.

Im Wintersemester 1966 bekam ich einen Studienplatz für Humanmedizin in Erlangen. An der Friedrich-Alexander-Universität Erlangen-Nürnberg legte ich 1967 das Vorphysikum und 1969 das Physikum ab.

Am 5. Juli 1972 bestand ich das medizinische Staatsexamen mit der Note "sehr gut".

